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Research Paper

Strong Mucosal and Systemic Immunities Induced by Nasal Immunization with Anthrax Protective Antigen Protein Incorporated in Liposome–Protamine–DNA Particles

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Purpose. The very lengthy and complicated dosing schedule of the current anthrax vaccine adsorbed, which was licensed in the USA for the prevention of cutaneous anthrax infection, calls for the development of an efficacious and easily administrable vaccine to prevent against the most lethal form of anthrax infection, the inhalation anthrax. We propose to develop a nasal anthrax vaccine using anthrax protective antigen (PA) protein carried by liposome–protamine–DNA (LPD) particles.
Methods. PA was incorporated in LPD particles and nasally dosed to mice. The resulting PA-specific immune response and lethal toxin neutralization activity were measured.
Results. Mice nasally immunized with PA incorporated into LPD particles developed both systemic and mucosal anti-PA responses. The anti-PA immunities induced included the production of anti-PA antibodies (IgG and IgM in the serum and IgA in nasal and lung mucosal secretions) and the proliferation of splenocytes after *in vitro* stimulation. The anti-PA IgG subtype induced was mainly IgG1. Finally, anthrax lethal toxin neutralization activity was detected both in the serum and in the mucosal secretions.
Conclusions. The anti-PA immune response induced by nasal PA incorporated in LPD was comparable to that induced by nasal PA adjuvanted with cholera toxin or subcutaneously injected PA adjuvanted with aluminum hydroxide.

KEY WORDS: lethal toxin neutralization; mucosa; Th2 response; vaccine delivery.

INTRODUCTION

Anthrax is an often-fatal bacterial infection caused by a rod-shaped, nonmotile, gram-positive bacterium *Bacillus anthracis* (1,2). Inhalation anthrax infection is the most lethal anthrax, with a death rate of close to 100% (3). It involves massive bacteremia and toxemia with unnoticed initial symptoms until the onset of hypotension, shock, and sudden death (1,4,5). Although *B. anthracis* strains are sensitive to many antibiotics, such as ciprofloxacin, the lack of early symptoms and the rapid course of the disease make antibiotic therapy practically ineffective for an inhalation anthrax infection. Thus, there is a need for an efficacious vaccine against inhalation anthrax. The only anthrax vaccine currently licensed in the USA is the AVA, an aluminum hydroxide (Alum)-adsorbed, formalin-treated culture supernatant of a toxigenic *B. anthracis* strain (6). AVA has many limitations, including the difficulty in maintaining consistency, the relatively high rate of local and systemic side effects, and most importantly, a very complicated and lengthy dosing schedule. The vaccination begins with a series of subcutaneous injections occurring over 18 months with subsequent annual boosters (6,7). Moreover, AVA was originally licensed only for the prevention of cutaneous anthrax, and its efficacy for inhalation anthrax prevention in humans has yet to be confirmed.

Anthrax is a toxin-mediated disease. Anthrax toxin contains three components: the protective antigen (PA), the lethal factor (LF), and the edema factor (EF). Individually, these components are not toxic. However, they can combine to form two binary toxins: the lethal toxin (LeTx, i.e., LF + PA) and the edema toxin (ET, i.e., EF + PA). PA binds to a cell-surface anthrax toxin receptor (ATR) and mediates the entry of LF and EF into the cytosol of host cells (8). LF and EF are only toxic when they are inside the cells (2). Thus, Abs that neutralize PA block the transport of both LF and EF into the cytosol and, in doing so, block the course of infection. The only demonstrable protective component in the AVA is the PA. Anti-PA IgG, actively induced or passively administered, had been shown to confer protection to animals challenged with *B. anthracis* either by aerosol or by intravenous injection, demonstrating that anti-PA Abs play an important role in the immunity against *B. anthracis* (9,10). Moreover, anti-PA Abs have been shown to recognize spore-associated proteins, to stimulate spore uptake by macrophages, and to interfere with the germination of spores *in vitro* (11,12). Thus, many recent anthrax vaccine developments have focused on the production of a subunit vaccine based on the PA protein.

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To protect against inhaled anthrax spore infection, such as what happened in the fall of 2001, an efficacious anthrax vaccine that can be readily used for mass immunization in the event of an emergency is needed. A nasal anthrax vaccine could be a good candidate because it will be simpler to administer than the AVA injectable. In addition, because inhaled anthrax spores enter hosts through the respiratory mucosal surface, it is conceivable that a nasal anthrax vaccine that can induce PA-specific immune responses both in the systemic compartment and in the mucosa of the respiratory tract will be more effective. Finally, because of their antispore activity (11,12), anti-PA Abs generated in the mucosal secretions of the respiratory tract should theoretically be able to bind to inhaled spores and prevent them from entering into hosts, and thus provide improved immunity against inhalation anthrax. In support of this, recent data showed that, in rabbits and mice nasally immunized with PA, serum toxin-neutralization activity (TNA) alone was not predictive of the survival of animals after an inhalation anthrax challenge (13,14), which was in contrast to the previous belief that serum TNA was predictive of the effectiveness of anthrax vaccines (10,15).

The nasal mucosa is an important arm of the mucosal system. It has (i) an epithelial compartment on the surface of the epithelium and the underlying connective tissue that contains immunocompetent cells, such as macrophages, dendritic cells (DCs), and intraepithelial lymphocytes; (ii) the organized lymphoid structure of nose-associated lymphoid tissues (NALTs); and (iii) the lymph nodes (LNs) draining the respiratory system (16). The Waldeyer's ring, which includes the nasopharyngeal tonsil or adenoid, the pair of palatine tonsils, the pair of tubal tonsils, and the inguinal tonsils, is present in the human pharynx (17). NALT is a well-organized structure, consisting of B and T cells areas, which are covered by an epithelial layer containing M cells (18). Because of the lack of afferent lymphatics, exogenous antigens are sampled directly from the mucosal surface covering the NALT to a large extent by M cells (19). The DCs, which are rich in the epithelial tissues, may also pick up antigens present on the mucosal surface (20). Thus, the nasal mucosa is expected to be an excellent site to administer vaccine, which was clearly demonstrated by the launch of the FluMist® into the market.

The feasibility of inducing anti-PA immune response by nasal PA protein has been previously confirmed (13,14,21,22). Although nasal administration of PA alone cannot induce any detectable anti-PA immune response, entrapment of PA in poly(lactic-co-glycolic acid) (PLGA) microspheres or liposomes (soy PC), or coadministration of PA with an adjuvant, such as cholera toxin (CT) and CpG motif-containing oligos, enabled the nasally administrated PA to induce anti-PA immune responses in murine models (13,14,21,22). Boyaka *et al.* (22) detected anthrax lethal TNA in mucosal secretions, when mice were nasally dosed with PA adjuvanted with CT. Although CT is a powerful mucosal adjuvant (23), its severe toxicity precludes its potential use in humans (24). Currently, the feasibility of replacing the wild-type CT with its detoxified form is being tested. Mikszta *et al.* (14) also detected specific anti-PA Abs in the lung lavages of mice nasally immunized with PA adjuvanted with CpG motif-containing oligos. However, it is known that the adjuvanticity data of CpG oligos obtained in murine are difficult to be transferred to humans because of the significant difference that exists in their DC biology (25). Nevertheless, there continues to be a need for the development of an efficacious PA-based nasal vaccine.

Previously, we have reported a liposome-based particle, named liposome–polycation–DNA (LPD), as a vaccine carrier for subcutaneous administration of protein-based vaccines (26), although the LPD particles *per se* were not an effective delivery system for DNA vaccine (27). LPD was engineered by combining cationic liposomes with protamine-condensed DNA. Upon mixing, the components spontaneously rearrange to form virus-like particles with the condensed DNA inside the liposomes. An antigen of interest may be incorporated into these LPD particles simply by adding the antigen into the preparation prior to the spontaneous rearrangement (26). We hypothesized that nasal dosing of PA protein incorporated into LPD particles will induce strong anti-PA immune responses both in the peripheral blood and in mucosal secretions, especially the respiratory tracts, of immunized mice.

MATERIALS AND METHODS

Preparation of PA Protein Incorporated LPD Particles

Liposomes and LPD were prepared as previously described (26). Briefly, small unilamellar liposomes composed of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, Avanti Polar Lipids, Inc., Alabaster, AL, USA)/cholesterol (Sigma-Aldrich, St. Louis, MO, USA, molar ratio 1:1) were prepared by a thin-film hydration method followed by membrane extrusion (0.1 μm). The DOTAP concentration was fixed at 10 mg/ml. The LPD was composed of DOTAP/cholesterol liposomes, protamine (Sigma), and an empty plasmid DNA (pNGVL from the National Gene Vector Laboratory, Indianapolis, IN, USA) in a ratio of 9.0:0.6:1.0 (w/w/w). Plasmid pNGVL is a DNA vector engineered for the construction of DNA vaccine. It has CpG motifs with appropriate flanking sequences. The plasmid was amplified in *Escherichia coli* and was purified with an Endo-Free plasmid purification kit from Qiagen (Valencia, CA, USA). To prepare LPD, liposomes (21.5 μl, 10 mg/ml) and protamine (7.5 μl, 2 mg/ml) were dispersed in 75 μl of aqueous solution containing 10% dextrose (Sigma). Seventy-five microliters of aqueous solution containing pNGVL (50 μg) and recombinant protective antigen (rPA) (30 μg) was added dropwise into the mixture of liposomes and protamine while gently shaking. The mixture was allowed to stay at room temperature for at least 15 min prior to further use. The incorporation efficiency for the rPA in LPD was estimated to be ~80% using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). In SDS-PAGE gel, unincorporated rPA moved into the gel, whereas those incorporated remained in the loading well. A comparison of the intensity of the rPA band from rPA/LPD preparation with that when the same amount of pure rPA was loaded in a different well in the same gel allowed us to roughly estimate the incorporation efficiency. The size of the rPA-incorporated LPD particles was determined using photon correlation spectroscopy to be around 200 nm.

Nasal Immunization

Female Balb/C mice (6–8 weeks, 18–20 g, Charles River Laboratories, Inc., Wilmington, MA, USA) were nasally immunized once a week for three consecutive weeks with 5 μg of rPA (List Biological Laboratories, Inc., Campbell, CA, USA) in 25 μl of LPD suspension. For nasal immunization, mice were lightly anesthetized with pentobarbital (7 mg/100 g, Abbott Laboratories, North Chicago, IL, USA) and were given a total volume of 25 μl of rPA/LPD, in two doses of 12.5 μl with 10–15 min between each dose. As controls, mice ($n = 5$) were either left untreated, nasally (i.n.) dosed with rPA alone in phosphate-buffered saline (PBS, 10 mM, pH 7.4), nasally dosed with rPA adjuvanted with cholera toxin (CT, 1 μg/mouse, List Biological Laboratories), or subcutaneously (s.c.) injected with rPA (5 μg/mouse) admixed with Alum (aluminum hydroxide gel, USP, Spectrum Chemical and Laboratory Products, New Brunswick, NJ, USA, 15 μg/mouse). Mouse blood and lung and nasal washes were collected 28 days after the first immunization. This immunization experiment was repeated two times.

To collect nasal and lung washes, an incision was made in the trachea of euthanized mice. The nasal wash was collected by pipetting 0.2 ml of sterile PBS (10 mM, pH 7.4) through the trachea toward the nose. Fluid was collected in a microfuge tube as it exited the nares. The lung wash was obtained by pipetting 0.4 ml of PBS into the trachea toward the lungs, aspirating back into the pipette tip, and reinjecting once before the final withdrawal of the PBS fluid from the lung. The samples were stored at −80°C prior to further use.

ELISA for Anti-PA Ab Measurement

The levels of anti-PA Abs were determined using enzyme-linked immunosorbent assay (ELISA). Briefly, enzyme immunoassay/radioimmunoassay flat-bottom, medium-binding, polystyrene 96-well plates from Corning Costar (Corning, NY, USA) were coated at 4°C overnight with 100 ng of rPA dissolved in 0.1 M carbonate buffer (pH 9.6). Plates were washed with PBS/Tween 20 (10 mM, pH 7.4, 0.05% Tween 20) and were blocked with 4% (w/v) bovine serum albumin (BSA; Sigma) in PBS/Tween 20 for 1 hr at 37°C. Serum samples were diluted in 4% BSA/PBS/Tween 20, added to the plates, and incubated for 2.5 hr at 37°C. Goat–anti-mouse immunoglobulin (IgG, IgA, IgM, IgG1, or IgG2a) conjugated to horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) was added as secondary Ab. The presence of bound Ab was detected following a 30-min incubation at room temperature in the presence of 3,3′,5,5′-tetramethylbenzidine substrate (TMB, Sigma). The reaction was stopped by adding sulfuric acid (Sigma, 0.2 M). The absorbance was read at 450 nm using a SpectraMax plate reader (Molecular Devices Inc., Sunnyvale, CA, USA). The levels of anti-PA IgA in the lung and nasal washes were also determined similarly.

The sample dilution was completed by 2- or 10-fold serial dilutions. In cases of serial 2-fold dilutions, specific Ab level was reported as titer (anti-$\log_2$). Otherwise, it was reported as the OD450 after the TMB solution was incubated at room temperature for 30 min.

Lethal Toxin-Neutralization Activity Assay

Toxin-neutralization activity was determined as described elsewhere with modifications (22). Briefly, confluent J774A.1 cells were seeded (5×10^4 cells/well) in sterile, 96-well, clean-bottom plates (Corning Costar) and incubated at 37°C, 5% CO_2 for 12 hr. A fresh solution containing 40 ng/ml LF (List Biological Laboratories) and 400 ng/ml rPA was mixed with an equal volume of diluted serum or mucosal wash samples in triplicate and incubated for 1 hr at 37°C. The mixture was then added to J774A.1 cells and incubated at 37°C, 5% CO_2 for 3 hr. Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit (Sigma), with untreated cells and LeTx-alone treated cells as controls.

Splenocyte Proliferation Assay

Single splenocyte suspension was prepared as previously described (28). Splenocytes from individual spleens were cultured at a density of 4×10^6 cells/ml and stimulated with rPA (0 or 12.5 μg/ml) in complete Roswell Park Memorial Institute (RPMI) medium (Invitrogen, Carlsbad, CA, USA) for 5 days at 37°C, 5% CO_2 (22). Boyaka *et al.* (22) reported 12.5 μg/ml of rPA as the optimal concentration to induce

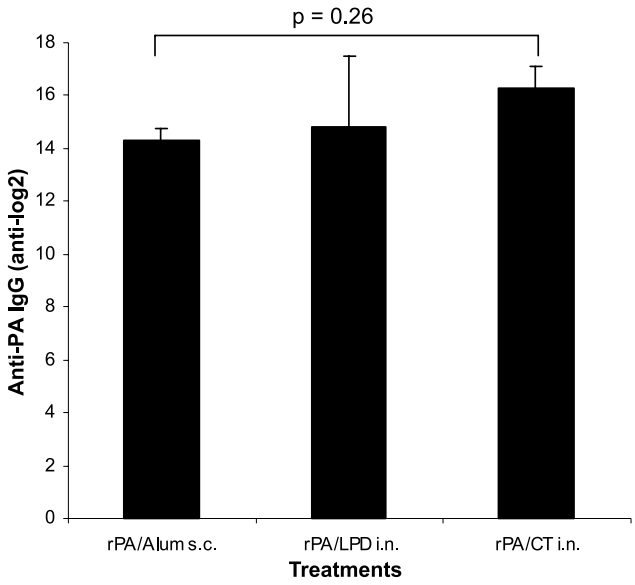

Fig. 1. Serum anti-PA Ab responses. Balb/C mice ($n = 5$) were nasally dosed with recombinant protective antigen (rPA) alone, rPA-incorporated liposome–protamine–DNA (rPA/LPD), or left untreated on days 0, 7, and 14. As controls, other two groups of mice ($n = 5$) were nasally dosed with rPA adjuvanted with cholera toxin (rPA/CT i.n., CT = 1 μg/mouse) or s.c. injected with rPA adjuvanted with Alum (rPA/Alum s.c., Alum = 15 μg/mouse). The dose of rPA was 5 μg/mouse. On day 28, mice were bled, and total anti-PA IgG titer in the serum was determined using enzyme-linked immunosorbent assay (ELISA) with the serum being diluted 2-fold serially. Nasal rPA alone failed to induce any detectable level of anti-PA IgG. Analysis of variance (ANOVA) showed that there was not any significant difference among those three treatments ($p = 0.26$). This experiment was repeated twice, and similar results were obtained. Data from one representative are shown.

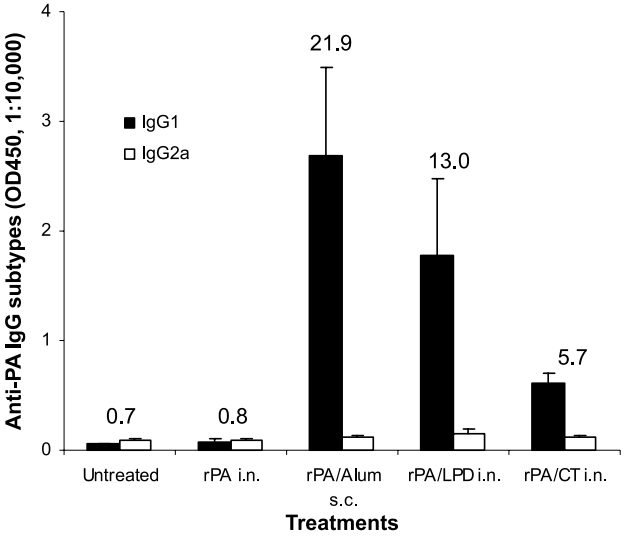

Fig. 2. Serum anti-PA IgG subtypes. Balb/C mice ($n = 5$) were dosed with rPA alone, rPA/LPD, or left untreated on days 0, 7, and 14. As controls, other groups of mice were injected (s.c.) with rPA adjuvanted with Alum or nasally dosed with rPA adjuvanted with CT. The IgG1 and IgG2a levels in the serum were determined on day 28 using ELISA after the sera were diluted 10,000-fold. The ratio of the OD450 for IgG1 and IgG2a ($OD450_{IgG1}/OD450_{IgG2a}$) was reported as numbers above the bars. Statistical analysis did not reveal any significant difference among all the values for IgG2a ($p = 0.13$, ANOVA). The IgG1 values from rPA/Alum (s.c.), rPA/LPD (i.n.), and rPA/CT (i.n.) were all significantly higher than that from untreated mice.

splenocyte proliferation. Cell proliferation was determined using an MTT kit.

Statistics

Statistical analyses were completed using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference procedure. A p value of ≤ 0.05 (two-tailed) was considered to be statistically significant.

RESULTS

Nasal Immunization of Mice with rPA-incorporated LPD Particles (rPA/LPD) Induced Strong Anti-PA Ab Responses in the Serum

As previously reported, nasal rPA alone did not induce any significant anti-PA IgG production (data not shown). However, nasal immunization of mice with rPA/LPD induced a high level of anti-PA IgG in their sera, which was comparable to that induced by nasal rPA adjuvanted with CT or s.c. rPA adjuvanted with aluminum hydroxide (Fig. 1). Serum anti-PA IgG was detectable 15 days after the first immunization (data not shown). Nasal rPA/LPD and nasal rPA adjuvanted with CT also induced anti-PA IgM in mouse sera, whereas IgM was not detected in the serum of mice injected (s.c.) with rPA adjuvanted with Alum (data not shown).

The Anti-PA Response was Th2-biased

To evaluate the T helper (Th) cell response, the level of IgG subtypes, IgG1 and IgG2a, in the serum was measured using ELISA. Similar to injection (s.c.) of rPA adjuvanted with Alum, nasal rPA/LPD induced a high level of anti-PA IgG1, but not IgG2a, suggesting that the immune response induced was biased toward Th2 (Fig. 2). As reported by Boyaka *et al.* (22), Ab response induced by nasal rPA adjuvanted with CT was also biased toward Th2.

Specific Anti-PA IgA was Induced in both the Nasal and Lung Washes

To investigate the mucosal immune response induced, the levels of anti-PA IgA in the nasal and lung washes were measured. As shown in Fig. 3, nasal rPA/LPD induced anti-PA IgA in both nasal and lung washes. In fact, the level of mucosal anti-PA IgA induced by nasal rPA/LPD was comparable to that induced by nasal rPA adjuvanted with CT. In contrast, no anti-PA IgA was detected in the nasal and lung washes of mice s.c. injected with rPA adjuvanted with Alum.

Splenocytes Isolated from Mice Nasally Immunized with rPA/LPD Proliferated After *In Vitro* Stimulation with rPA Protein

To investigate whether a proliferative immune response was induced by our nasal rPA/LPD vaccine, splenocytes

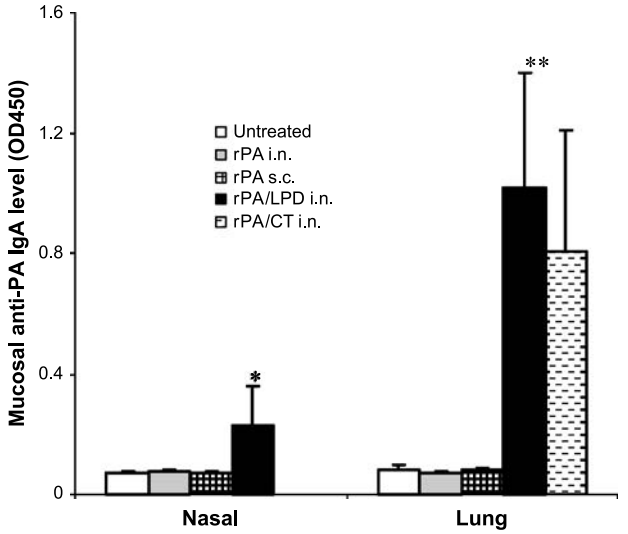

Fig. 3. Anti-PA IgA in nasal and lung washes. Balb/C mice were dosed (i.n.) with rPA alone, rPA/LPD, or left untreated on days 0, 7, and 14. As controls, other groups of mice were injected (s.c.) with rPA adjuvanted with Alum or nasally dosed with rPA adjuvanted with CT. On day 28, mice were sacrificed. Both nasal and lung washes were collected. Anti-PA IgA level in samples from individual mouse was determined using ELISA after the washes were diluted 10-fold. * indicates that, for nasal washes, the value for rPA/LPD (i.n.) was significantly higher than that of the others. Nasal washes were not collected from mice nasally dosed with rPA/CT. ** indicates that, for lung washes, the values from rPA/LPD (i.n.) and rPA/CT (i.n.) were not different from each other, but significantly higher than that from the others.

isolated from immunized mice were stimulated with rPA for 5 days, and the cell proliferation was measured. As shown in Fig. 4, although not as strong as the proliferation of splenocytes isolated from mice nasally immunized with rPA adjuvanted with CT, significant splenocyte proliferation was observed in mice nasally immunized with rPA/LPD. As expected, splenocytes isolated from mice injected (s.c.) with rPA adjuvanted with Alum also proliferated significantly.

Nasal rPA/LPD Vaccine Induced Protective Lethal Toxin-Neutralization Activity

To evaluate the protective activity of the anti-PA Abs induced, the anthrax LeTx neutralization activity in the serum and mucosal wash samples was measured using an *in vitro* macrophage (J774A.1) protection assay. As shown in Fig. 5A, strong LeTx neutralization activity was induced in the serum of mice nasally immunized with rPA/LPD. In fact, the LeTx neutralization activities in the sera of mice nasally immunized with rPA/LPD, nasally immunized with rPA adjuvanted with CT, and s.c. injected with rPA adjuvanted with Alum were comparable. LeTx neutralization activity was also detected in the nasal and lung washes of mice nasally immunized with rPA/LPD or rPA adjuvanted with CT (Fig. 5B and data not shown). In contrast, no neutralization activity was detected in the nasal and lung washes of mice nasally dosed with rPA alone or s.c. injected with rPA adjuvanted with Alum.

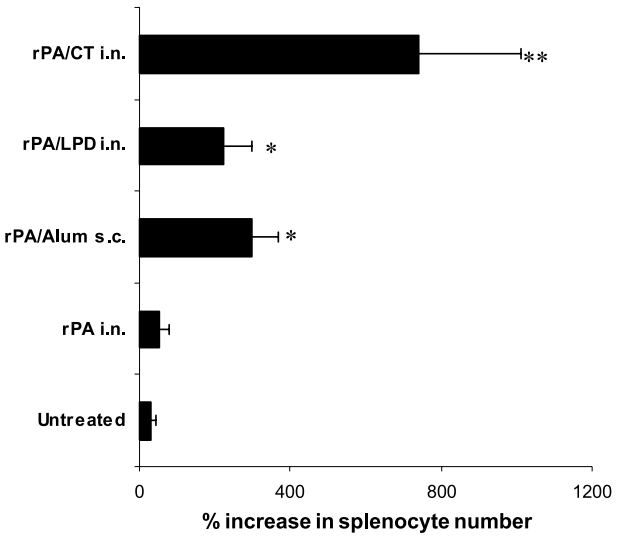

Fig. 4. *In vitro* proliferation of splenocytes after stimulation with rPA. Balb/C mice were dosed (i.n.) with rPA alone, rPA/LPD, or left untreated on days 0, 7, and 14. As controls, other groups of mice were injected (s.c.) with rPA adjuvanted with Alum or nasally dosed with rPA adjuvanted with CT. On day 28, mice were sacrificed. Their spleens were harvested, and single splenocyte suspension from individual spleen was prepared. The splenocytes (4×10^6/ml) were incubated with rPA (0 or 12.5 μg/ml) for 5 days. The proliferation was measured using an MTT test kit. ANOVA showed that there was a significant difference among the values from different treatments ($p \ll 0.05$). * indicates that the value for rPA/Alum (s.c.) was not different from that for rPA/LPD (i.n.), but higher than that from the untreated mice and mice nasally dosed with rPA alone. ** indicates that the value for rPA/CT (i.n.) was significantly higher than that of the others.

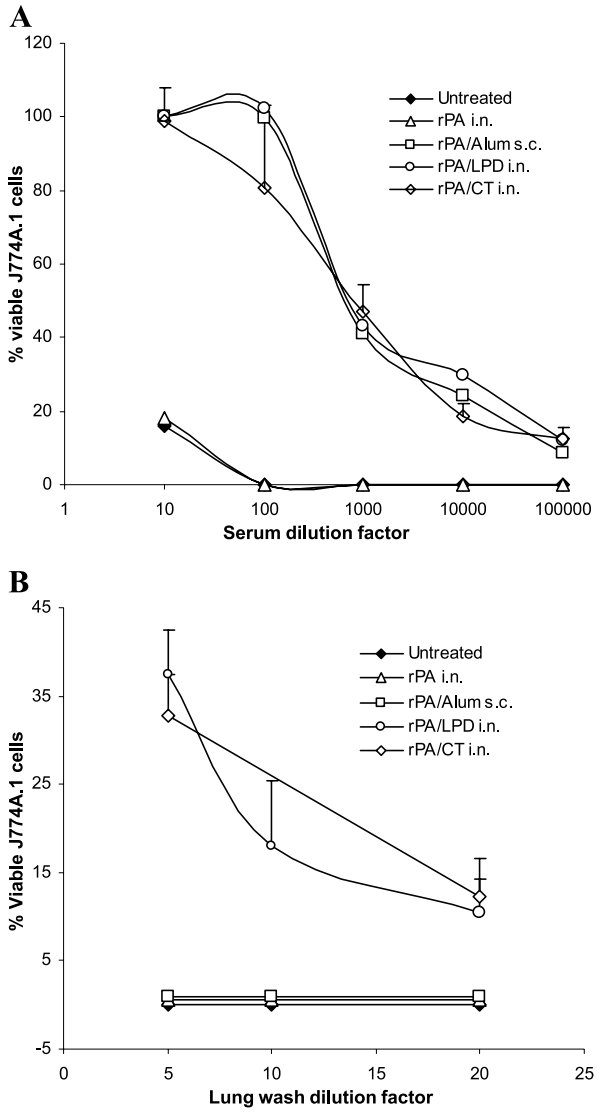

Fig. 5. Nasal rPA/LPD induced strong LeTx neutralization activity in serum (A) and lung washes (B). Balb/C mice ($n = 5$) were dosed as mentioned in Materials and Methods. On day 28, sera were collected. The 10-fold serial dilutions of each serum samples were incubated with J774A.1 cells (5×10^5/ml) in the presence of anthrax lethal toxin (LeTx, 400 ng/ml of PA and 40 ng/ml of LF) for 3 h. The percent of viable J774A.1 cells was determined using an MTT test. In (A), only the standard deviations from rPA/CT (i.n.) were included to show that the LeTx neutralization activity induced by rPA/LPD (i.n.), rPA/Alum (s.c.), and rPA/CT (i.n.) were not significantly different from one another. In (B), toxin-neutralization activity was not detected in the lung washes of untreated mice and mice nasally immunized with rPA alone or s.c. injected with rPA adjuvanted with Alum. The data for rPA/CT (i.n.) after 10-fold dilution were not collected.

DISCUSSION

Although cutaneous and gastrointestinal anthrax infections have produced documented fatalities, inhalation anthrax is the most lethal form of anthrax, with a fatality rate close to 100%, if proper antibiotic intervention is not provided (3). Upon inhalation of anthrax spores by a susceptible host, the spores are taken up locally by phagocytic cells. The

phagocytized spores are either cleared or carried to regional LNs, where surviving spores germinate and outgrow (11,29,30). The vegetative bacilli proliferate, spread systemically, and secrete anthrax toxins (11). An antispore immune response that interferes early in this sequence of events, before the onset of bacteremia and toxemia, could conceivably provide more effective protection against a lethal anthrax infection (11). Anti-PA Abs were shown to have antispore activities (11,12). For example, spore-associated proteins recognized by anti-PA Abs were shown to be present on the surface of spores (11). Moreover, anti-PA Abs were also shown to stimulate the phagocytosis of spores by macrophages and inhibit the germination of spores *in vitro* (11). Thus, it is conceivable that a nasal PA protein-based vaccine can be an excellent candidate to induce anti-PA Ab responses both in the mucosa of respiratory tract to block anthrax spore uptake and germination and in the serum to neutralize anthrax toxins resulted from bacteremia. Moreover, a nasal anthrax vaccine is expected to be more patient-friendly to administer and potentially more cost-effective than a sterile injectable.

Results in this study clearly demonstrated that protective anti-PA immunities were induced in the mucosal secretions of mice when they were nasally immunized with rPA protein incorporated in our LPD particles. We showed that anti-PA IgA was induced in the nasal and lung washes of mice nasally immunized with rPA/LPD (Fig. 3). More importantly, the mucosal anti-PA immune response was shown to have LeTx neutralization activity in a J774A.1 macrophage protection assay (Fig. 5B). Based on some recently published data, we speculate that this strong LeTx-neutralization anti-PA immunity, which was comparable in strength to that induced by nasal rPA adjuvanted with CT, will provide a certain extent of antispore activity, and thus interfere with the early stage of inhalation anthrax infection. We will confirm this activity in future studies. Mikszta *et al.* (14) reported that in rabbits immunized nasally with rPA adjuvanted with CpG oligos, serum toxin neutralization Ab titers alone were not predictive of the survival of rabbits after an aerosol anthrax spore challenge, which was in contrast to the previously reported correlation between the effectiveness of an anthrax vaccine and the serum TNA induced by it when AVA or other rPA-based vaccine was injected intramuscularly (10,15). The authors suggested that it was possible that the nasal route of vaccine dosing may provide stronger local responses at mucosal surfaces, thus enabling better protection from an aerosol challenge, despite the lower serum TNA titers. Similarly, using rPA-loaded PLGA microspheres, Flick-Smith *et al.* (13) reported that mice immunized with a combination of i.m. and nasal priming and boosting schedules were always fully protected against aerosol anthrax spore challenges, whereas i.m. injection of the PA-loaded microsphere alone was not as effective, emphasizing the importance of stimulating both mucosal and systemic immune systems to obtain a full protection.

Strong anti-PA Abs were also induced in the systemic compartment of mice nasally immunized with our rPA/LPD vaccine (Figs. 1, 4, and 5A). Anti-PA IgG titers higher than 1000 was detected in mouse serum 15 days after the first dose (data not shown). On day 28, the serum anti-PA IgG titer was comparable to that induced by nasal rPA adjuvanted with CT and s.c. rPA adjuvanted with Alum (Fig. 1). The splenocytes isolated from mice nasally dosed with rPA/LPD also proliferated significantly after *in vitro* stimulation with rPA protein (Fig. 4). Although the types of the splenocytes that proliferated still need to be identified in future studies, based on the Th2-biased Ab response as suggested by the high ratio of IgG1/IgG2a (13.0 ± 6.5), it was likely that $CD4^+$ T cells proliferated. The LeTx neutralization activity in the serum of mice nasally immunized with rPA/LPD was comparable to that in mice s.c. injected with rPA/Alum or nasally immunized with rPA adjuvanted with CT (Fig. 5A). Thus, we expect our nasal rPA/LPD vaccine to be at least as effective as rPA adjuvanted with the other two standard vaccine adjuvants (CT and aluminum hydroxide) in protecting against an anthrax challenge. Meanwhile, our nasal rPA/LPD is advantageous in that, in comparison to CT, which has severe toxicity, LPD has an established good safety profile in a recent clinical trial (31). In our studies, 40% of mice nasally dosed with CT (1 μg/mouse) died during the experiments, whereas no mortality was observed in any other mice dosed similarly without CT. In addition, nasal rPA/LPD is more convenient and patient-friendly to administer than the s.c. injection of rPA adjuvanted with Alum. In future studies, we will evaluate the protective activity of nasal rPA/LPD in pulmonary anthrax spore-challenging studies and further elucidate the relationship between the presence of anti-PA Abs in the mucosal secretion of the respiratory tract and the effectiveness of a vaccine against an inhalation anthrax spore challenge in mice.

In the current study, the rPA dose per mouse was only 5 μg. Boyaka *et al.* (22) previously reported that rPA alone was not immunogenic when dosed nasally. However, when CT was used as an adjuvant, there was a correlation between the dose of nasal rPA and the resulting anti-PA Ab response, with 40 μg/mouse being the optimal dose (22). Further increase of the dose of rPA to above 40 μg/mouse did not lead to higher anti-PA Ab response. The anti-PA IgG titer in mice nasally dosed with 40 μg rPA was more than 100-fold higher than that in mice nasally dosed with 10 μg of rPA (22). If this holds true for our nasal rPA/LPD, we expect that the anti-PA IgG titer induced by our nasal rPA/LPD with 40 μg of rPA will be at least comparable with that induced by 40 μg of nasal rPA adjuvanted with CT. We will evaluate the effect of rPA dose in our nasal rPA/LPD on the resulting anti-PA immune response in future studies.

We have also evaluated the anti-PA IgG subtypes induced by our nasal rPA/LPD vaccine, which suggested that a Th2-biased antibody response was induced (Fig. 2) because the IgG1/IgG2a ratio obtained was 13.0 ± 6.6, in comparison to the 0.6 ± 0.1 observed in the naive mice. The high level of anti-PA IgG1 induced is important because it was shown that the anti-PA IgG1 subtype has a high affinity to PA protein (32). A Th2-biased immune response is also beneficial for the development of an anthrax vaccine because anthrax is a toxin-mediated disease, and a Th2 response aids in the production of Abs. However, it is counterintuitive to observe that the anti-PA Ab response was biased toward Th2 because there were plasmids in our LPD particles, and the unmethylated CpG motifs in bacterial plasmid DNA usually skew the resulting immune response toward Th1 when the DNA is

intramuscularly injected into mice (33,34). In fact, in our previous studies, we found that the pNGVL and the cationic liposomes in the LPD both had adjuvant activity, and that the induced immune response was Th1-biased when the LPD was subcutaneously injected into mice (26,35). The nasal route of administration may be responsible for the Th2-biased response observed in the current study. It is known that the immune response from DNA vaccine usually biases toward Th1 after i.m. injection (33). However, the response is usually biased toward Th2 when DNA vaccine is intradermally (i.d.) injected using gene gun (33). In addition, the low dose of DNA applied to mice using gene gun was thought by some to be responsible for the Th2-biased response (34). The dose of pNGVL applied nasally was about 4 μg/mouse, which was a relatively low dose and may explain the observed Th2-biased response. Finally, the Th2-biased Ab response may also suggest that the adjuvanticity from the plasmid DNA in our rPA/LPD vaccine may not be required for it to induce the anti-PA immune response, which is not surprising because our previous studies showed that the plasmid DNA in the LPD was not absolutely required for LPD to be immunostimulatory (35). Replacement of the CpG motif-containing plasmid with CpG motif-free oligos did not significantly change the immunostimulatory activity of the LPD (unpublished data). We will exploit this possibility in future studies by using rPA/LPD prepared with CpG motif-free oligos. The dispensability of the plasmid DNA in the rPA/LPD can be advantageous because it will eliminate the uncertainty for transferring the success of rPA/LPD in mice to humans.

Mechanistically, the immunogenicity of rPA/LPD could be mainly caused by its particulate nature, which can be readily taken up by M cells in the NALTs when compared to rPA protein in solution. In nasal immunization, it is known that the nasally dosed vaccine has a great potential to be inhaled into the lung. Lung involvement usually led to a stronger specific immune response, but can potentially cause serious proinflammatory response in the lung, and thus limit its use in humans (19,36,37). With our dosing technique and FITC-labeled rPA/LPD, we have confirmed that nasally dosed FITC-labeled rPA/LPD mainly remained in the nare passages 4 h after dosing, while it was not detectable in the lung washes (data not shown), suggesting that the contribution from lung immunization to the induced PA-specific immune response was limited. As to the potential involvement of gut-associated lymphoid tissue (GALT) in inducing anti-PA immune response, it is difficult to speculate to what extent our rPA/LPD had traveled to the gastrointestinal tract after nasal dosing. However, we tend to believe that GALT involvement was insignificant because it is known that a very large dose is generally required for an orally dosed vaccine to induce any significant immune response.

As mentioned earlier, a few recent publications have described nasal dosing of rPA protein as an antigen in animal models (13,14,21,22). However, our nasal rPA/LPD is advantageous not only because of the strong mucosal and systemic anti-PA immune responses induced by it but also because of the established good safety profile of LPD. When systemically injected via the i.v. route, LPD was shown to be transiently inflammatory by producing cytokines (27). LPD had been intraventricularly administered to children with Canavan disease for gene therapy in a previous clinical trial and was shown to be well tolerated (31). In the present studies, we did not observe any gross inflammatory, allergic, or toxic effect when mice were nasally dosed with our rPA/LPD. In fact, the high anti-PA IgG1/IgG2a ratio suggested that Th1-driving proinflammatory cytokines (e.g., IFN-γ and IL-12) observed after i.v. injection of LPD were not induced after nasal dosing with rPA/LPD. Finally, as mentioned earlier, LPD was prepared via a spontaneous rearrangement process after cationic liposomes and protamine/DNA complex were mixed together. Also, LPD particles can be lyophilized and stored at room temperature for months (38). Thus, we believe this nasal rPA/LPD vaccine holds a great potential to be developed into a human anthrax nasal vaccine.

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